

Cross-reactive IgE antibody responses to tropomyosins from *Ascaris lumbricoides* and cockroach

Ana Beatriz R. Santos, PhD,^a Gutemberg M. Rocha, MD,^b Constance Oliver, PhD,^b Virgínia P. L. Ferriani, MD,^c Rodrigo C. Lima, MD,^a Mário S. Palma, PhD,^d Valéria S. F. Sales, MD,^e Rob C. Aalberse, PhD,^f Martin D. Chapman, PhD,^g and L. Karla Arruda, MD^a *Ribeirão Preto, Rio Claro, and Natal, Brazil, Amsterdam, The Netherlands, and Charlottesville, Va*

Background: Evidence indicates that infection with *Ascaris lumbricoides* may promote development of allergy and asthma. **Objective:** To study the role of tropomyosin, a pan-allergen in invertebrates, in IgE responses to *A lumbricoides*.

Methods: Recombinant *A lumbricoides* and *Periplaneta americana* tropomyosins were expressed in *Pichia pastoris*. Levels of IgE to tropomyosins from *A lumbricoides* and *P americana* were determined by chimeric ELISA in sera from 119 children living in a parasite-endemic area and 112 patients with cockroach allergy from the allergy clinics. Presence of tropomyosin in *A lumbricoides* larvae at L3 stage was evaluated by immunofluorescence using mAb 1A6, directed against mite tropomyosin. Molecular modeling of *P americana* and *A lumbricoides* tropomyosins was performed by using the MODELLER program.

Results: *A lumbricoides* tropomyosin showed 69% to 98% sequence identity to tropomyosins from other invertebrates. The predicted structure of *A lumbricoides* tropomyosin was similar to that of *P americana* tropomyosin and showed the characteristic coiled-coil structure. Strong correlation was found for IgE antibodies to tropomyosins from *A lumbricoides* and *P americana* in sera from children living in a parasite-endemic area and from patients with cockroach allergy. Larvae of *A lumbricoides* reacted strongly with mAb 1A6.

Conclusion: Tropomyosin induces IgE responses in *A lumbricoides*-infected children and in patients allergic to cockroach. (J Allergy Clin Immunol 2008;121:1040-6.)

Key words: Tropomyosin, *Ascaris lumbricoides*, *Periplaneta americana*, IgE cross-reactivity, allergy, parasites, asthma

It is estimated that 2 billion individuals worldwide are currently infected with the intestinal parasites *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, and *Strongyloides stercoralis*.¹ Similar to patients with allergy, individuals infected with helminths often develop increased secretion of T_H2-type cytokines, eosinophilia, and high levels of IgE.² In addition, certain parasite infections, particularly those with *Schistosoma mansoni*, have been shown to trigger regulatory responses with increased IL-10 production, which may underlie the observation of inhibition of atopy and development of allergic symptoms in infected individuals.³⁻⁵ Studies performed in an endemic area of schistosomiasis in Brazil revealed reduced skin prick test reactivity to inhalant allergens, and an attenuated course of asthma in individuals harboring *S mansoni* eggs in their feces.^{6,7} However, the protective role of parasite infection in allergy and asthma may not be generalized to all species. Previous reports and a recent meta-analysis revealed that current infection with *A lumbricoides* was associated with a significant increase in the risk of asthma,⁸⁻¹⁰ whereas infection with hookworm was associated with a reduction in risk.¹⁰

Intervention studies aimed at clarifying the role of parasite infections in allergy and asthma have provided conflicting results.⁴ An open-label randomized controlled trial performed in Gabon showed that antihelminthic treatment of children with a high prevalence of infection, using praziquantel and mebendazole every 3 months, resulted in increased skin test reactivity to house dust mites.¹¹ In Venezuela, an open-label randomized study demonstrated that monthly treatment with albendazole over a period of 1 year reduced symptoms of wheeze and the need for asthma medications.¹² More recently, a cluster-randomized study in Ecuador revealed that treatment of a large group of children currently infected by *A lumbricoides*, *T trichiura*, *Ancylostoma duodenale*, or *S stercoralis* with albendazole every 2 months for 12 months was highly effective in reducing helminth infection, but it was not associated with increase in the prevalence of atopy or allergy symptoms.¹³

We have focused our studies on the role of tropomyosin in IgE responses to *A lumbricoides*. Tropomyosins are highly conserved proteins that have been shown to be pan-allergens in invertebrates including shrimp and other crustaceans and mollusks, mites, and cockroach.¹⁴ In addition, tropomyosin has been identified in *Anisakis simplex*, a fish parasite that can cause allergic reactions

From the Departments of ^aInternal Medicine, ^bCell and Molecular Biology and Pathogenic Bioagents, and ^cPediatrics, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto; ^dthe Biosciences Institute, State University of São Paulo, Rio Claro; ^ethe Federal University of Rio Grande do Norte, Natal; ^fSanquin Research at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam; and ^gIndoor Biotechnologies, Inc, Charlottesville.

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Reprint requests: L. Karla Arruda, MD, School of Medicine of Ribeirão Preto Av Bandeirantes, 3900 Ribeirão Preto, SP, 14049-900, Brazil. E-mail: karla@fmrp.usp.br. 0091-6749/\$34.00

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Abbreviation used

GM: Geometric mean

in human beings.¹⁵ The high degree of amino acid sequence identity among invertebrate tropomyosins provides support for immunological cross-reactivity,¹⁶ and some studies have suggested that this cross-reactivity may be clinically relevant.¹⁷ Sensitization and allergic symptoms after ingestion of snails and shrimp have been reported after specific immunotherapy with house dust mite,^{18,19} and increased severity of allergic reactions to snails has been observed in children receiving specific immunotherapy with dust mite extracts.²⁰ IgE antibody reactivity to shrimp among Orthodox Jews, who are prohibited from eating shellfish, is thought to be a result of tropomyosin.²¹

Preliminary data from our laboratory revealed that a mAb directed against mite tropomyosin showed strong binding to *A lumbricoides* striated muscle tissue from adult worms by means of immunofluorescence.²² In the current study, we have determined the amino acid sequence of *A lumbricoides* tropomyosin, expressed recombinant tropomyosins from *A lumbricoides* and from the cockroach species *P americana*, and compared IgE antibody responses to both proteins in patients with asthma and/or rhinitis allergic to cockroach, and in children living in an endemic area for *A lumbricoides*.

METHODS

Subjects

A panel of sera from 112 patients with asthma, wheezing, and/or rhinitis, age 2 to 52 years, who participated in previous studies from our group²³⁻²⁵ was analyzed for the presence of IgE antibodies to *P americana* and *A lumbricoides* tropomyosin. All patients presented allergy to cockroach, defined by presence of a positive skin test to extracts of *P americana* and/or *Blattella germanica* and/or the presence of specific IgE to *B germanica* in serum (ImmunoCap class ≥ 2). Sera from 119 children age 3 to 6 years who attended a day care center in Natal, Northeast Brazil, an endemic area for *A lumbricoides*, were also analyzed. Twenty-nine of these children participated in a previous study on the role of parasitic infection in allergy and asthma.⁹ Four subjects without allergy served as controls for assays for IgE antibodies to tropomyosin. The study was approved by the Ethics Committee of the School of Medicine of Ribeirão Preto.

Identification of cDNA coding for tropomyosin in *A lumbricoides*

Total RNA was extracted from 100 mg of an adult *A lumbricoides* worm by using Trizol LS (Invitrogen, Carlsbad, Calif). DNA amplification was performed by RT-PCR, using primers synthesized on the basis of the sequences of *P americana* tropomyosin (Genbank AF106961)²³ and *Anisakis simplex* tropomyosin (Genbank Y19221).¹⁵ Previous sequencing of partial cDNA coding for *A lumbricoides* tropomyosin, performed in our laboratory, using primers based on the DNA coding for *P americana* tropomyosin conserved sequences (MDAIKKK and LKEAETRAE), revealed that tropomyosin from *A lumbricoides* presented 98% identity to *A simplex* tropomyosin. Primer sequences were as follows: forward primer 5' ATGGAMGCGATCAAGAAG 3', where M represents A or C; reverse primer 5' ATATCCGGAAAGTTCCTTGAA 3'. RT-PCR was performed as previously described.²⁶ Briefly, 10 μ L *A lumbricoides* RNA was mixed with deoxynucleotides 20 μ mol/L, reverse primer 20 μ mol/L, dithiothreitol 0.1 mol/L, reverse transcriptase 0.5 μ L (Invitrogen, 200 U/ μ L), and recombinant RNase Out Ribonuclease Inhibitor (Amersham Pharmacia, Uppsala, Sweden, 20,000 U/mL) 0.5 μ L for 10 minutes at room temperature, and incubated at 37°C for 1 hour and at 95°C for 10 minutes. The forward primer was added, followed by 3 minutes incubation at 95°C. Reactions were performed in 50 μ L volume, with denaturation at 95°C for

1.5 minute, annealing at 55°C for 1.5 minute, extension at 72°C for 1 minute for 34 cycles, and a final 10-minute step at 72°C using Taq polymerase 0.25 μ L (Invitrogen, 5 U/ μ L). Amplified DNA was ligated into pCR2.1 vector (TA cloning kit; Invitrogen) and prepared for sequencing (QIAGEN Plasmid Purification system).

Production of recombinant tropomyosins from *A lumbricoides* and *P americana*

A lumbricoides RNA was used as a template to generate an 879-bp product for cloning into pPIC9 *Pichia pastoris* vector (Invitrogen), and DNA amplification was performed by RT-PCR as described. Primers for PCR were synthesized as follows: 5' GCGCTACGTAATGGACGCGATCAAG AA 3' (sense), containing a *SnaB* I restriction site, and 5' ATAAGAATGCGGCCGCATATCCG GAAAGTTCTT 3' (antisense), containing a *Not* I restriction site. Digested PCR products were ligated into *SnaB* I and *Not* I sites of pPIC9, and expression of recombinant proteins in the *P pastoris* system (Invitrogen) was performed following the manufacturer's instructions. Plasmid DNA encoding *P americana* tropomyosin identified from a UNI-Zap expression library²³ (Stratagene, La Jolla, Calif) was used as a template to generate a 864-bp PCR product for cloning into the pPIC9. Primers for PCR were synthesized as follows: 5' GCGC TACGTAATGGACGCGATCAAGAA 3' (sense), and 5' ATAAGAATGCGG CCGCTTAGTTGCCAATAAGTT 3' (antisense), containing *SnaB* I and *Not* I restriction sites, respectively. After an initial denaturation step of 5 minutes at 95°C, PCR incubations were performed for 1 minute at 55°C and 3 minutes at 72°C for 30 cycles followed by a final extension at 72°C for 15 minutes.

Cultures were grown at 30°C with shaking, with addition of a 0.5% final concentration of methanol every 24 hours, and samples were collected at 24, 48, 72, and 96-hour time points for analysis. *Pichia*-expressed tropomyosins from *A lumbricoides* and *P americana* were purified from culture supernatants over separate mAb affinity columns, using anti-tropomyosin mAb 1A6. This mAb was originally raised against *Dermatophagoides pteronyssinus* tropomyosin; however, it recognizes tropomyosins from cockroach,²³ mites, and shrimp.²⁷ Twenty-five milligrams of mAb 1A6 were coupled to 1 g cyanogen bromide-activated Sepharose (Amersham Pharmacia), and culture supernatants were applied to the columns at 4°C. Proteins were eluted with 0.005 mol/L glycine pH 2.8 and analyzed by silver-stained SDS-PAGE using 8% to 25% gradient gels (Pharmacia Phast System).

Molecular modeling of tropomyosins from *A lumbricoides* and *P americana*

The MODELLER program²⁸ was used for modeling of tropomyosins from *A lumbricoides* and *P americana* with the α -carbon atomic coordinates from porcine tropomyosin (Protein Data Bank code 1C1G). Spatial restraints and CHARMM energy terms²⁹ were combined into an objective function. A total of 250 models were generated for each protein, and optimized final models were selected on the basis of stereochemical quality, assessed by the program PROCHECK.³⁰ The cutoff for hydrogen bonds and salt bridges was 3.4 Å.

Immunofluorescence analysis of *A lumbricoides* larvae at stage L3

Eggs were obtained from adult worms of *A lumbricoides* by dissection and cultivated for 4 weeks. Eggs containing larvae at the L3 stage were concentrated by centrifugation at 1500 rpm for 10 minutes, immersed in tissue-freezing medium (Tissue Tek; Electron Microscopy Sciences, Hatfield, Pa), and frozen with dry ice and acetone. Ten-micrometer sections were placed on glass slides, rinsed 5 times with PBS, and blocked with 2% BSA-PBS for 15 minutes at room temperature. Sections were incubated for 1 hour with mAb 1A6 (5 μ g/mL), rinsed 5 times with PBS, and incubated for 30 minutes with goat anti-mouse IgG conjugated to AlexaFluor 594 (25 μ g/mL; Molecular Probes; Invitrogen). Slides were rinsed 8 times with PBS, mounted with Fluormount-G (Electron Microscopy Sciences), and examined by using a Nikon Ultraphot FX microscope (Nikon Instruments, Melville, NY). Slides incubated without primary antibody or with unrelated primary antibody, mAb 1D8, directed to mite allergen Der p 2 (2.5 μ g/mL), served as controls.

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1  ATGGACGCGATCAAGAAGAAGATCGAGCGATGAAATCGAAGGACAATGCATTGGAT  60
   M D A I K K K M Q A M K I E K D N A L D
61  CGTGCGGATGCTGCCAAGAGAAGGTGCGCGAGATGACGATAAGCTCGAGCGCATTTGAG  120
   R A D A A E E K V R Q M T D K L E R I E
121 GAGGAACCTTCGAGATACCAAGAAATGATGCAAGCCGAAGACGACCTCGATAAGGCA  180
   E E L R D T Q K K M M Q T E N D L D K A
181 CAAGAAGATCTGCGGTTCGTAATTCGAATCTCGAAGAGAGAGAAGAAAGTCCAAGAG  240
   Q E D L S V A N S N L E E K E K K V Q E
241 GCGAGGCGAGAAGTTGCTGCTTTGAATCGTATGACGCTTTTGAAGAGGAACCTGGAA  300
   A E A E V A A L N R R M T L L E E E L E
301 CGAGCTGAAGAAGCTTTGAAATTTGCCACCGAGAACTCGAAGAGCAACACATACTGCT  360
   R A E E R L K L A T E K L E E A T H T A
361 GACGAATCTGAGCGTGTGCGCAAGGTGATGAGAACCGCTCATTCCAAGATGAGGAACGT  420
   D E S E R V R K K V M E N R S F Q D E E R
421 GCGAACCCTCGAATCACAGCTCAAGAAGTTCAGATGCTTGAAGAAGCTGATCGC  480
   A N T V E S Q L K E V Q M L A E E A D R
481 AAATACGACGAGTTTGCCTCCCAATTTGGCAATGGTTGAGGCTGATCTGGAACGAGCTGAA  540
   K Y D E V A R K L A M V E A D L E R A E
541 GAACCTCGACAGGCTGGCGAAGACAGATCGTGCAGCTGGAAGAGGAATTCGCTGTTGTC  600
   E R A E A G E N K I V E L E E E L R V V
601 GCGAAGCAACCTGAATCTCTCGAAGTTCCTCGAAGAGGACACTACAACGAGAGACTCA  660
   G N N L K S L E V S E E K A L Q R E D S
661 TACGAAGAAGATCGCGCATCTTTTCGCGACGCTTAAAGGAGGCTGAGACTCGCGCGGAA  720
   Y E E Q I R T V S A R L V K E A E T R A E
721 TTTCGCGAAGATCAGTGCAGAAATTCGAGAAAGAGTGCAGACTTGAAGACGAGCTG  780
   F A E R S V Q K L Q K E V D R L E D E L
781 GTACAGGAGAAAGACATACAGAGATCTCTCGGAGAACTTGATCAACCTTCCAGAA  840
   V H E K E R Y K S I S E E L D Q T F Q E
841 CTITCCGATAT 852
   L S G Y

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FIG 1. Nucleotide and deduced amino acid sequence of *A lumbricoides* tropomyosin. The sequence comprised 852 base pairs, coding for a 284-amino acid protein. The estimated molecular mass of *A lumbricoides* tropomyosin was 33.2 kd. No potential N-linked glycosylation sites were identified within the sequence.

Measurements of specific IgE to *A lumbricoides* and *P americana* tropomyosin

Levels of IgE to *A lumbricoides* and *P americana* tropomyosin were measured by chimeric ELISA.³¹ Briefly, microtiter plates were coated with 1 µg/well mAb 1A6 overnight at 4°C in carbonate-bicarbonate buffer, pH 9.6. After washing, plates were incubated with recombinant tropomyosin from *A lumbricoides* and *P americana* (rPer a 7) at 0.5 µg/mL. Sera were added at 1:10 dilution, followed by incubation with biotinylated goat antihuman IgE (1:4000) and streptavidin-peroxidase (1:1000). The reaction was developed by using 1 mmol 2,2'-azino-bis(3-ethylbenzthiazoline-6 sulphonic acid) (Sigma, St Louis, Mo) and H₂O₂, and the assay was quantitated by using a chimeric mouse Fab/human Fc epsilon antibody (clone 2B12-IgE).^{31,32}

Sequencing analysis

DNA sequencing was performed by using the automated ABI 100 Model 377 system (University of Virginia, Charlottesville), and similarity searches were performed with the BLAST program. Amino acid sequencing was performed by using a gas-phase sequencer PPSQ-21 A (Shimadzu, Kyoto, Japan) based on automated Edman Degradation Chemistry.

Statistical analysis

Levels of IgE to *A lumbricoides* and *P americana* tropomyosin were compared by linear regression analysis using log-transformed data. The GraphPad Prism 4.0 program (GraphPad Software Inc, San Diego, Calif) was used for statistical analysis and for construction of graphs.

RESULTS

The nucleotide sequence coding for *A lumbricoides* tropomyosin and the deduced amino acid sequence are shown in Fig 1. The sequence of the 284-amino acid protein showed 90% to 98% identity to tropomyosins from other parasites, including *A simplex*, and 74% and 69% identity to mite and cockroach tropomyosins, respectively. The estimated molecular mass of *A lumbricoides* tropomyosin was 33.2 kd; both the DAIKKK (EAIKKK) N-terminal motif and the L-K-E-A-E-x-R-A-E signature sequences were present in *A lumbricoides* tropomyosin (Fig 2).

Molecular models derived from the sequences of *A lumbricoides* and *P americana* tropomyosins showed the characteristic coiled-coil structure common to tropomyosins (Fig 3).

Recombinant *P americana* tropomyosin (Per a 7 allergen) was expressed in *P pastoris* culture supernatants at 24 hours, with maximum production in 72 to 96 hours (data not shown). After purification, SDS-PAGE analysis revealed a 68-kd band under nonreducing conditions and a 34-kd band, corresponding to monomeric protein, under reducing conditions. These results suggested that the 68-kd protein corresponded to a homodimer of tropomyosin (Fig 4, A). The yield was 7 mg purified protein/L culture. *A lumbricoides* tropomyosin was expressed as a recombinant protein by using a similar method, with a yield of 0.22 mg purified protein/L culture. Analysis of recombinant tropomyosin revealed major bands of estimated molecular weights of 30 kd and 38 kd (Fig 4, B). Amino acid sequencing of a peptide comprising residues 11 to 32, identified in both higher-molecular-weight bands, and of a peptide comprising residues 224 to 233, obtained from the lower-molecular-weight band, confirmed the identity of the *A lumbricoides* recombinant protein to tropomyosin and suggested that the protein had been cleaved into several large peptides during expression. Addition of protease inhibitors in expression cultures has not modified this apparent protease activity (data not shown). N-terminal sequencing showed that both recombinant proteins lacked the first 10 amino acid residues; however, this region of the molecule has not previously been identified as containing IgE epitopes.¹⁶

Forty-seven of the 112 patients from the allergy clinic (42%) had IgE to Per a 7 and to *A lumbricoides* tropomyosin. Levels of IgE antibodies to Per a 7 varied from 0.4 to 300 IU/mL (geometric mean [GM], 1.0 IU/mL) and to *A lumbricoides* tropomyosin ranged from 0.4 to 900 IU/mL (GM, 0.9 IU/mL). Among the 119 children living in Northeast Brazil, 90 (75.6%) and 93 (78.1%) subjects presented IgE to Per a 7 and to *A lumbricoides* tropomyosin, respectively. Levels of IgE antibodies to Per a 7 varied from 0.4 to 175 IU/mL (GM, 2.1 IU/mL) and to *A lumbricoides* tropomyosin ranged from 0.4 to 325 IU/mL (GM, 2.4 IU/mL). There was a significant correlation of levels of IgE to *A lumbricoides* and *P americana* tropomyosin in sera of patients from Ribeirão Preto ($r = 0.97$; $P < .0001$) and of children from Natal ($r = 0.95$; $P < .0001$; Fig 5, A and B). Patients seen in the allergy clinics with positive IgE to tropomyosin presented no differences of severity of asthma or rhinitis symptoms compared with those not sensitized to tropomyosin. Likewise, *Ascaris*-infected children with detectable IgE to tropomyosin showed no differences in the frequency of wheezing or lung problems compared with those negative for IgE to this protein (data not shown).

The fact that tropomyosin from *Ascaris* can react with antitropomyosin antibodies from other species was demonstrated by the strong reactivity of mAb 1A6, an antibody raised against mite tropomyosin, to *A lumbricoides* L3-stage larvae (Fig 6, A). Control sections stained without the primary antibody or with an unrelated antibody (anti-Der p 2 mAb; Fig 6, B) showed only nonspecific fluorescence of the egg outer membrane.

DISCUSSION

Tropomyosin from the parasite *A lumbricoides* presents a high degree of sequence identity to tropomyosins from other

	1				50
<i>A. lumbricoides</i>	MDAIIKKMQA	MKIEKDNALD	RADAAEEKVR	QMTDKLERIE	EELRDTQKKM
<i>A. simplex</i>	MDAIIKKMQA	MKIEKDNALD	RADAAEEKVR	QMTDKLERIE	EELRDTQKKM
<i>D. pteronyssinus</i>	MEATIKKMQA	MKLEKDNAID	RAEIAEQKAR	DANLRAEKSE	EEVRLAQKKI
<i>P. americana</i>	MDAIIKKMQA	MKLEKDNAMD	RALLCEQQAR	DANLRAEKAE	EEARSLQKKI
	51				100
<i>A. lumbricoides</i>	MQTENDLDKA	QEDLSVANSN	LEEKEKKVQE	AAEVAALNR	RMTLLEEELE
<i>A. simplex</i>	MQTENDLDKA	QEDLSTANSN	LEEKEKKVQE	AAEVAALNR	RMTLLEEELE
<i>D. pteronyssinus</i>	QQIENELDQV	QEQLSAANTK	LEEKEKALQT	AEGDVAALNR	RIQLIEEDLE
<i>P. americana</i>	QQIENELDQT	MEQLMQVNAK	LDEKDKALQN	AESEVAALNR	RIQLIEEDLE
	101				150
<i>A. lumbricoides</i>	RAEERLKLAT	EKLEEATHTA	DESERVVKVM	ENRSFQDEER	ANTVESQLKE
<i>A. simplex</i>	RAEERLKLAT	AKLEEATHTA	DESERVVKVM	ENRSFQDEER	ANTVESQLKE
<i>D. pteronyssinus</i>	RSEERLKIAT	AKLEEASQSA	DESERMKML	EHRSTIDEER	MEGLENQLKE
<i>P. americana</i>	RSEERLATAT	AKLAEASQAA	DESERARKIL	ESKGLADEER	MDALENQLKE
	151				200
<i>A. lumbricoides</i>	AQMLAEADR	KYDEVARKLA	MVEADLERAE	KRAEAGENKI	VELEELRVV
<i>A. simplex</i>	AQMLAEADR	KYDEVARKLT	MVEADLERAE	ERAETGENKI	VELEELRVV
<i>D. pteronyssinus</i>	ARMMAEDADR	KYDEVARKLA	MVEADLERAE	ERAETGESKI	VELEELRVV
<i>P. americana</i>	ARFMAEADK	KYDEVARKLA	MVEADLERAE	ERAESGESKI	VELEELRVV
	201				250
<i>A. lumbricoides</i>	GNNLKSLEVS	EEKALQREDS	YEEQIRTVSA	RLKEAETRAE	FAERSVQKLQ
<i>A. simplex</i>	GNNLKSLEVS	EEKALQREDS	YEEQIRTVSA	RLKEAETRAE	FAERSVQKLQ
<i>D. pteronyssinus</i>	GNNLKSLEVS	EEKAQOREEA	HEQQIRIMTT	KLKEAEARAE	FAERSVQKLQ
<i>P. americana</i>	GNNLKSLEVS	EEKANLREEE	YKQIKTLTT	RLKEAEARAE	FAERSVQKLQ
	251				284
<i>A. lumbricoides</i>	KEVDRLEDEL	VHEKERYKSI	SEELDQTFQE	LSGY	
<i>A. simplex</i>	KEVDRLEDEL	VHEKERYKSI	SEELDQTFQE	LSGY	
<i>D. pteronyssinus</i>	KEVGRLEDEL	VHEKERYKSI	SDELDQTFAE	LTGY	
<i>P. americana</i>	KEVDRLEDEL	VHEKERYKFI	CDDLDMTFTE	LIGN	

FIG 2. Sequence alignment of *A lumbricoides* tropomyosin to tropomyosins from nematode, mite, and cockroach. The deduced amino acid sequence from cDNA showed 98%, 73%, and 69% identity to tropomyosins from *A simplex* (GenBank Y19221.1), mite (GenBank Y14906), and cockroach (GenBank AF106961.1), respectively. The conserved N-terminal sequence DAIKKK is underlined, and the tropomyosin signature sequence L-K-E-A-E-x-R-A-E is indicated in *red*.

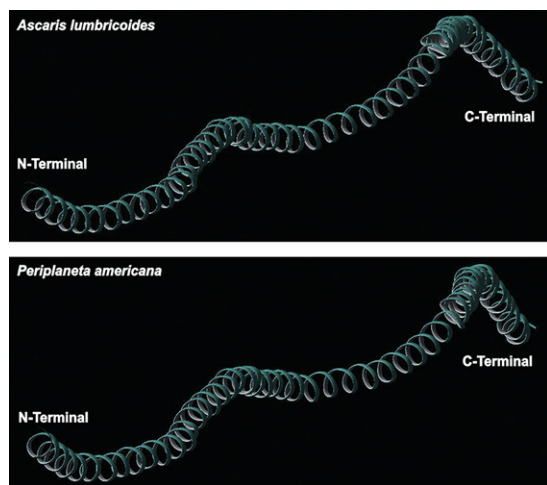


FIG 3. Molecular modeling of the 3-dimensional structure of *P americana* and *A lumbricoides* tropomyosin. Modeling was performed by using the coordinates of porcine tropomyosin. Tropomyosins from *A lumbricoides* and *P americana* showed the α -helical structure common to tropomyosins from other sources.

invertebrates, including cockroach, mites, and shrimp. Invertebrate tropomyosins share greater than 70% sequence identity, whereas comparisons of amino acid sequences of invertebrate tropomyosins and vertebrate tropomyosins, which are nonallergenic, reveal a degree of 51% to 58% identity.¹⁶ Molecular modeling revealed that *A lumbricoides* tropomyosin shares the typical α -helical, coiled-coil structure with tropomyosins from other sources.

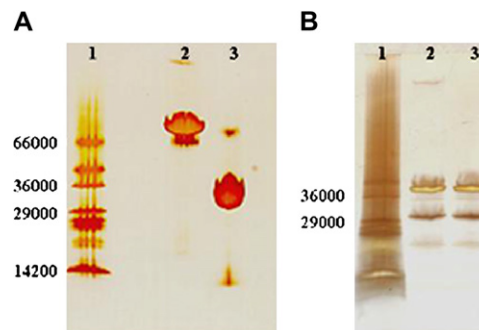


FIG 4. Expression of recombinant tropomyosins from *P americana* (A) and *A lumbricoides* (B). Proteins were analyzed by silver-stained SDS-PAGE. A, Lane 1: molecular weight markers; lanes 2 and 3: rPer a7 (0.35 mg/mL), under nonreducing and reducing conditions, respectively. B, Lane 1: molecular weight markers; lanes 2 and 3: recombinant *A lumbricoides* tropomyosin (0.22 mg/mL) under nonreducing and reducing conditions, respectively.

Tropomyosin is expressed in high levels in L3 stage *A lumbricoides* larvae, the stage of pulmonary passage of the parasite. Human IgE antibody binding to *A lumbricoides* tropomyosin and to cockroach tropomyosin from *P americana* showed a strong correlation.

Although both *A lumbricoides* and cockroach recombinant tropomyosins produced in the current study lacked the first 10 N-terminal amino acid residues because of cleavage during expression, we believe that the IgE binding activity of the dominant large peptides of up to 38 kD has not been decreased.

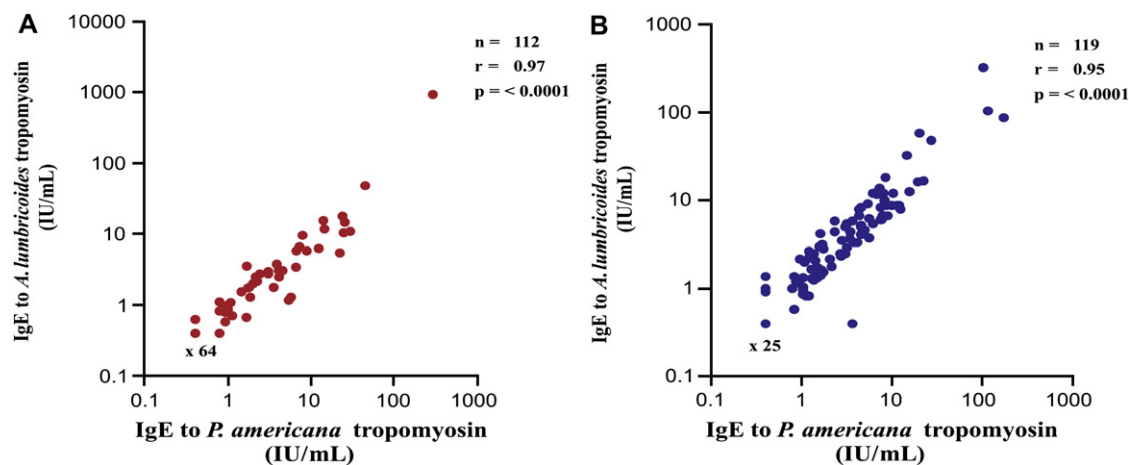


FIG 5. Levels of IgE to *A. lumbricoides* and *P. americana* tropomyosins. There was a significant correlation of levels of IgE to *A. lumbricoides* and *P. americana* tropomyosins in sera of patients with asthma and/or rhinitis from Ribeirão Preto (**A**) and in sera of children living in Natal (**B**), areas with low and high prevalence of *A. lumbricoides* infection, respectively.

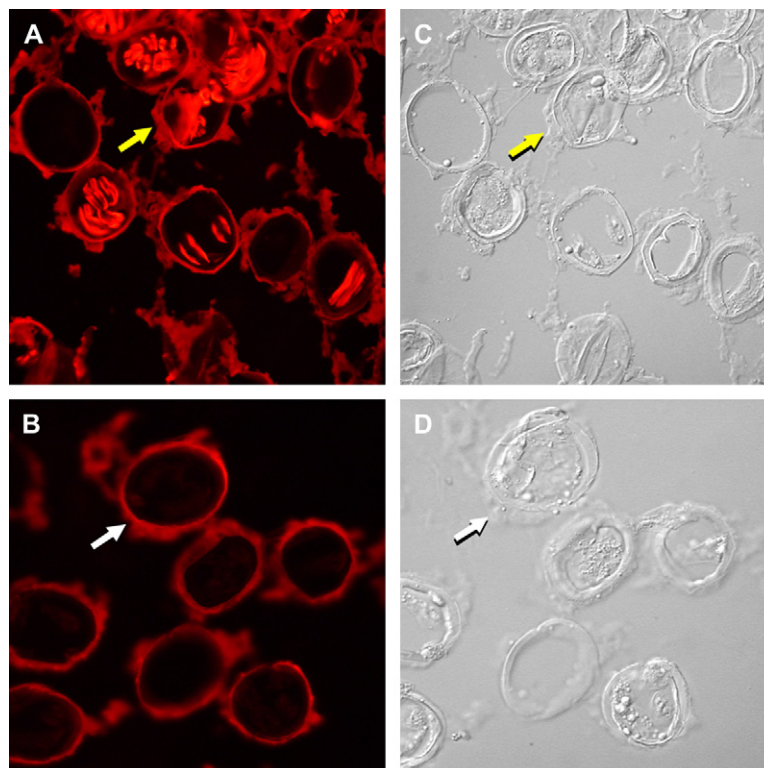


FIG 6. Reactivity of antitropomyosin mAb 1A6 with *A. lumbricoides* larvae. **A**, Strong reactivity of mAb 1A6 to L3 stage *A. lumbricoides* larvae was observed by immunofluorescence. **B**, Control sections stained with an unrelated antibody revealed nonspecific fluorescence of the egg outer membrane, despite presence of larvae. **C** and **D**, Differential interference contrast images of **A** and **B**.

Analysis of the *A. lumbricoides* tropomyosin sequence at regions of IgE binding epitopes previously identified in shrimp tropomyosin (Pen a 1),³³ which do not encompass the N-terminal portion of the molecule, showed 80% identity or greater to 5 of the 8 epitopes (see this article's Table E1 in the Online Repository at www.jacionline.org). In the study by Ayuso et al,³³ IgE

recognition of some of the cockroach tropomyosin (Per a 7) epitopes, particularly epitopes 2, 3a, 3b, 4, and 5a, by patients with shrimp allergy was similar to that of Pen a 1 epitopes. Our data suggest cross-reactivity caused by shared sequences within IgE binding epitopes. In the current study, none of the *A. lumbricoides*-infected children presented positive skin test results to

cockroach; likewise, none of the patients with cockroach allergy with asthma and/or rhinitis were currently infected with *A lumbricoides*. However, sera from these individuals presented IgE to tropomyosin from both *P americana* and *A lumbricoides*, and a strong correlation of levels of IgE to both proteins was observed.

Infection with *A lumbricoides* has been associated with lack of protection or even increased risk for allergen sensitization and asthma symptoms in some studies,⁸⁻¹⁰ whereas infections with *Schistosoma* and hookworm were shown to promote protection.^{2-7,10} Among preschool children living in Brazil, current infection with *A lumbricoides* was strongly and independently associated with wheezing.⁹ A study in China revealed that infection with *A lumbricoides* was associated with increased risk of childhood asthma and sensitization with inhalant allergens.⁸ In a community in South Africa, children with elevated *A lumbricoides* IgE had a higher frequency of allergic symptoms, atopic diseases, and positive skin prick test results to aeroallergens than those without elevated IgE to the parasite. The increased risk of atopic symptoms was present among those with negative tuberculin test results.³⁴ In young adults with asthma or rhinitis, no effect of *A lumbricoides* infection on skin test reactivity to allergens was observed; in addition, IL-10 production by PBMCs stimulated with *D pteronyssinus* antigens showed no difference in patients infected with *A lumbricoides* compared with noninfected patients.³⁵ On the other hand, studies in Ecuador and Vietnam have pointed to an inverse association of geohelminth infections, including those with *A lumbricoides*, and skin test reactivity to allergens, suggesting a protective effect against sensitization among children in endemic areas.³⁶⁻³⁸

The pulmonary phase of larval migration that occurs in the life cycle of some parasites including *A lumbricoides* and hookworms has been associated with wheezing, bronchial hyperresponsiveness, pneumonitis, pronounced pulmonary eosinophilia, elevated serum IgE, and production of eotaxin and macrophage inflammatory protein 1 α , in both human and experimental studies.^{39,40} *A lumbricoides* adult and larval-stage antigens induced increased proliferative responses in PBMCs of infected subjects compared with uninfected individuals, which were accompanied by increased expression of IL-4 and IL-5, with no differences in parasite-specific IL-10 production in the 2 groups.⁴¹ It is thought that this highly polarized T_H2 immune response in the lung mucosa could cause symptoms and even enhance allergic reactivity to environmental allergens. We speculate that presence of tropomyosin in *A lumbricoides* L3 larvae in the respiratory tissue early in life could enhance T_H2 polarized responses. In endemic areas, children often get infected with *Ascaris* through ingestion of parasite eggs before the first year of life. Most of the larvae passing through the lung tissue die locally, allowing release of antigens including tropomyosin to be taken up by antigen-presenting cells, which in turn undergo migration to regional lymph nodes to stimulate T_H2 responses and IgE production. Progressively these children also get exposed through the inhalation route to allergens derived from mites and cockroach, which share the highly homologous tropomyosin. It is possible that IgE responses to tropomyosin derived from inhalant allergens could be amplified or develop more promptly as a result of previous sensitization to *Ascaris* tropomyosin, triggering persistent lung inflammation.

The protective effect of hookworms could not be explained on the basis of direct effect on lung tissue. Infection with hookworms occurs later when children are able to walk independently and

become susceptible to infection by larvae penetrating the skin. Therefore, it has been suggested that the age at which a parasitic infection is acquired may be an additional determining factor for subsequent modulation of allergic responses to environmental allergens by parasites.^{4,10}

Schistosomes, the worms implicated in suppression of allergy, on the other hand, do not present a pulmonary passage of larvae during their life cycle, and strongly induce IL-10 production. Schistosome-specific lysophosphatidylserine, a lipid structure derived from eggs and adult worms, has been shown to activate Toll-like receptor 2, leading to development of mature dendritic cells with the capacity of inducing IL-10–producing regulatory T cells.⁴² In addition, *Schistosoma* tropomyosin shows only 58% sequence identity to *A lumbricoides* tropomyosin.

In conclusion, the results of the current study show that tropomyosin is an important protein that induces IgE response in *A lumbricoides*–infected populations and in patients allergic to cockroach. Our results provide evidence to support immunologic cross-reactivity of allergens derived from *A lumbricoides*, inhalants (arthropods), and foods (crustaceans and mollusks), and suggest that *A lumbricoides* tropomyosin may have a role in development of allergy and asthma. The clinical relevance of IgE cross-reactive responses to tropomyosin would be best investigated in the setting of a birth cohort study, following infants from birth to school age, designed to detect the initial infections with *Ascaris* or other parasites; the development of IgE antibody responses, including those directed to tropomyosin; and the onset of allergic symptoms, in the context of other risk factors for development of allergy and asthma, including respiratory viral infections, allergen and endotoxin exposure, and breast-feeding.

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Clinical implications: Structural similarities of invertebrate tropomyosins could account for cross-reactive IgE antibody responses. Presence of tropomyosin in *A lumbricoides* larvae at the stage of pulmonary passage could enhance sensitization to tropomyosins from inhalant sources and contribute to allergic lung inflammation.

REFERENCES

1. Crompton DWT. *Ascaris* and ascariasis. *Adv Parasitol* 2001;48:285-375.
2. Carvalho EM, Bastos LS, Araújo MI. Worms and allergy. *Parasite Immunol* 2006; 28:525-34.
3. Fallon PG, Mangan NE. Suppression of Th2-type allergic reactions by helminth infection. *Nat Rev Immunol* 2007;7:220-30.
4. Cooper PJ, Barreto ML, Rodrigues LC. Human allergy and geohelminth infections: a review of the literature and a proposed conceptual model to guide the investigation of possible causal associations. *Br Med Bull* 2006;79-80:203-18.
5. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 2003;3:733-44.
6. Araujo MI, Lopes AA, Medeiros M, Cruz AA, Sousa-Atta L, Sole D, et al. Inverse association between skin response to aeroallergens and *Schistosoma mansoni* infection. *Int Arch Allergy Immunol* 2000;123:145-8.
7. Medeiros M Jr, Figueiredo JP, Almeida MC, Matos MA, Araujo MI, Cruz AA, et al. *Schistosoma mansoni* infection is associated with a reduced course of asthma. *J Allergy Clin Immunol* 2003;111:947-51.
8. Palmer LJ, Celedón JC, Weiss ST, Wang B, Fang Z, Xu X. *Ascaris lumbricoides* infection is associated with increased risk of childhood asthma and atopy in rural China. *Am J Respir Crit Care Med* 2002;165:1489-93.

9. Sales VS, Rodrigues CE, Trombone APF, Lima RC, Santos ABR, Oliver C, et al. Infection with *Ascaris lumbricoides* in pre-school children: role in wheezing and IgE responses to inhalant allergens [abstract]. J Allergy Clin Immunol 2002; 109:S27.
10. Leonardi-Bee J, Pritchard D, Britton J. Asthma and current intestinal parasite infection: systematic review and meta-analysis. Am J Respir Crit Care Med 2006; 174:514-23.
11. van den Biggelaar AH, Rodrigues LC, van Ree R, van der Zee JS, Hoeksma-Kruize YC, Souverein JH, et al. Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. J Infect Dis 2004;189:892-900.
12. Lynch NR, Palenque M, Hagel I, DiPrisco MC. Clinical improvement of asthma after anthelmintic treatment in a tropical situation. Am J Respir Crit Care Med 1997;156:50-4.
13. Cooper PJ, Chico ME, Vaca MG, Moncayo AL, Bland JM, Mafla E, et al. Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. Lancet 2006; 367:1596-603.
14. Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. Int Arch Allergy Immunol 1999;119:247-58.
15. Asturias JA, Eraso E, Martinez A. Cloning and high level expression in *Escherichia coli* of an *Anisakis simplex* tropomyosin isoform. Mol Biochem Parasitol 2000;108: 263-7.
16. Reese G, Schickentanz S, Lauer I, Randow S, Lüttkopf D, Vogel L, et al. Structural, immunological and functional properties of natural recombinant Pen a 1, the major allergen of brown shrimp, *Penaeus aztecus*. Clin Exp Allergy 2006;36:517-24.
17. Purohit A, Shao J, Degreaf JM, vanLeeuwen A, van Ree R, Pauli G, et al. Role of tropomyosin as a cross-reacting allergen in sensitization to cockroach in patients from Martinique (French Caribbean Island) with a respiratory allergy to mite and a food allergy to crab and shrimp. Allerg Immunol (Paris) 2007;39:85-8.
18. van Ree R, Antonicelli L, Akkerdaas JH, Pajno GB, Barberio G, Corbetta L, et al. Asthma after consumption of snails in house-dust-mite allergic patients: a case of IgE cross-reactivity. Allergy 1996;51:387-93.
19. van Ree R, Antonicelli L, Akkerdaas JH, Garritani MS, Aalberse RC, Bonifazi F. Possible induction of food allergy during mite immunotherapy. Allergy 1996;51:108-13.
20. Pajno GB, Lagrutta S, Barberio G, Canonica GW, Passalacqua G. Harmful effect of immunotherapy in children with combined snail and mite allergy. J Allergy Clin Immunol 2002;109:627-9.
21. Fernandes J, Reshef A, Patton L, Ayuso R, Reese G, Lehrer SB. Immunoglobulin E antibody reactivity to the major shrimp allergen, tropomyosin, in unexposed Orthodox Jews. Clin Exp Allergy 2003;33:956-61.
22. Arruda LK, Vailes LD, Ferriani VPL, Santos ABR, Pomés A, Chapman MD. Cockroach allergens and asthma. J Allergy Clin Immunol 2001;107:419-28.
23. Santos ABR, Chapman MD, Aalberse RC, Vailes LD, Ferriani VPL, Oliver C, et al. Cockroach allergens and asthma in Brazil: Identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. J Allergy Clin Immunol 1999;104:329-37.
24. Camara AA, Silva JM, Ferriani VPL, Tobias KRC, Macedo IS, Padovani MA, et al. Risk factors for wheezing in a subtropical environment: role of respiratory viruses and allergen sensitization. J Allergy Clin Immunol 2004;113:551-7.
25. Silva JM, Camara AA, Tobias KR, Macedo IS, Cardoso MR, Arruda E, et al. A prospective study of wheezing in young children: the independent effects of cockroach exposure, breast-feeding and allergic sensitization. Pediatr Allergy Immunol 2005;16:393-401.
26. Rakes GP, Arruda E, Ingram JM, Hoover GE, Zambrano JC, Hayden FG, et al. Rhinovirus and respiratory syncytial virus in wheezing children requiring emergency care. IgE and eosinophil analyses. Am J Respir Crit Care Med 1999;159: 785-90.
27. Witteman AM, Akkerdaas JH, Van Leeuwen J, Van Der Zee JS, Aalberse RC. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mites and insects. Int Arch Allergy Immunol 1994;105:56-61.
28. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 1993;234:779-815.
29. Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. CHARMM: a program for macromolecular energy minimization and dynamics calculations. J Comp Chem 1983;4:187-217.
30. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 1993;26: 283-91.
31. Trombone AP, Tobias KRC, Ferriani VPL, Schuurman J, Aalberse RC, Smith AM, et al. Use of chimeric ELISA to investigate immunoglobulin E antibody responses to Der p 1 and Der p 2 in mite-allergic patients with asthma, wheezing and/or rhinitis. Clin Exp Allergy 2002;32:1-6.
32. Schuurman J, Perdok GJ, Lourens TE, Parren PW, Chapman MD, Aalberse RC. Production of a mouse/human chimeric IgE monoclonal antibody to the house dust mite allergen Der p 2 and its use for the absolute quantification of allergen-specific IgE. J Allergy Clin Immunol 1997;99:545-50.
33. Ayuso R, Reese G, Leong-Kee S, Plante M, Lehrer SB. Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. Int Arch Allergy Immunol 2002;129:38-48.
34. Obihara CC, Beyers N, Gie RP, Hoekstra MO, Fincham JE, Marais BJ, et al. Respiratory atopic disease, *Ascaris*-immunoglobulin E and tuberculin testing in urban South African children. Clin Exp Allergy 2006;36:640-8.
35. Ponte EV, Lima F, Araujo MI, Oliveira RR, Cardoso LS, Cruz AA. Skin test reactivity and Der p-induced interleukin 10 production in patients with asthma or rhinitis infected with *Ascaris*. Ann Allergy Asthma Immunol 2006;96:713-8.
36. Cooper PJ, Chico ME, Rodrigues LC, Ordóñez M, Strachan D, Griffin GE, et al. Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. J Allergy Clin Immunol 2003;111:995-1000.
37. Cooper PJ, Chico ME, Bland M, Griffin GE, Nutman TB. Allergic symptoms, atopy, and geohelminth infections in a rural area of Ecuador. Am J Respir Crit Care Med 2003;168:313-7.
38. Flohr C, Tuyen LN, Lewis S, Quinnell R, Minh TT, Liem HT, et al. Poor sanitation and helminth infection protect against skin sensitization in Vietnamese children: a cross-sectional study. J Allergy Clin Immunol 2006;118:1305-11.
39. O'Lorcain P, Holland CV. The public health importance of *Ascaris lumbricoides*. Parasitology 2000;121:S51-71.
40. Culley FJ, Brown A, Girod N, Pritchard DI, Williams TJ. Innate and cognate mechanisms of pulmonary eosinophilia in helminth infection. Eur J Immunol 2002;32: 1376-85.
41. Cooper PJ, Chico ME, Sandoval C, Espinel I, Guevara A, Kennedy MW, et al. Human infection with *Ascaris lumbricoides* is associated with a polarized cytokine response. J Infect Dis 2000;182:1207-13.
42. van der Kleij D, Latz E, Brouwers JFHM, Kruize YCM, Schmitz M, Kurt-Jones EA, et al. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J Biol Chem 2002;277:8122-9.

TABLE E1. Sequence identity of *A lumbricoides* tropomyosin to IgE epitopes previously identified in shrimp tropomyosin (allergen Pen a 1)^{E1}

Epitope no.	Amino acid residues in Pen a 1 sequence	Sequence	Identity (%)
1	43-55	VHNLQKRMQQLEN	46
2	87-101	ALNRRIQLLEEDLER	80
3a	137-141	DEERM	80
3b	144-151	LENQLKEA	62.5
4	187-197	ESKIVELEEEEL	91
5a	249-259	LQKEVDRLEDEL	100
5b	266-273	KYKSITDE	62.5
5c	273-281	ELDQTFSEL	88.8

Eight IgE-binding epitopes within Pen a 1 allergen have been identified by Ayuso et al,^{E1} with sera from patients allergic to Pen a 1 who presented severe allergic reactions within 1 hour after ingestion of shrimp. Alignment of Pen a 1 sequences to homologous epitopes in *A lumbricoides* revealed sequence identities of 80% or greater to 5 of the 8 epitopes.

REFERENCE

- E1. Ayuso R, Reese G, Leong-Kee S, Plante M, Lehrer SB. Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. *Int Arch Allergy Immunol* 2002;129:38-48.